American Journal of Hematology 54:314–320 (1997)

Aggressive Natural Killer Cell Lymphoproliferative Disorder Associated With Epstein-Barr Viral RNA

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Lymphoproliferative disorder of natural killer cells is a heterogeneous disorder, and an association with Epstein-Barr virus (EBV) is suggested in some cases. A Japanese male presenting with recurrent nasopharyngeal problems developed fever, generalized lymphadenopathy, and hepatosplenomegaly. Separated cells from lymph nodes were shown to have a natural killer (NK) cell, CD2(+), CD3(−), CD16(+), CD56(+), HLA-DR(+) phenotype. A progressive abnormality of hepatic function was associated with hepatorenal failure and death. A serologic study suggested reactivated EBV infection. In situ hybridization (ISH) studies showed Epstein-Barr virus-encoded RNA (EBER)-1 in lymph nodes, with lymphocytes infiltrating the liver and tissue from ethmoid sinus surgery 3 years prior to development of obvious lymphoproliferative disease. Polymerase chain reaction performed on lymph node DNA, using oligonucleotide primers specific for the EBV lymphocyte-determined membrane antigen (LYDMA) gene, revealed a single band, suggesting monoclonal proliferation of the tumor. NK activities of the lymphocytes from the lymph node and peripheral blood were markedly decreased. These findings suggest a close relationship between EBV infection and development of NK cell lymphoproliferative disorder. Am. J. Hematol. 54:314–320, 1997.

Key words: Epstein-Barr virus-encoded RNA 1; in situ hybridization; natural killer cell; lymphoproliferative disorder

INTRODUCTION

Natural killer (NK) cells, which mediate nonmajor histocompatibility complex (MHC)-restricted lysis of tumor and virus-infected cells without prior sensitization, are found in 10–15% of peripheral blood lymphocytes that have a large granular lymphocyte (LGL) morphology [1–3]. Surface phenotyping of these LGLs has confirmed that they often express one or more of the NK cell-associated surface markers CD16, CD56, or CD57. In addition, a small subset of T cells (namely CD8+, presumably cytotoxic T cells) exhibits LGL morphology [4].

Infection with EBV is associated with lymphoproliferative disease of granular lymphocytes [5,6]. We recently observed a patient who died of rapidly deteriorating hepatorenal function and ileus soon after presenting with fever and cervical lymphadenopathy. Positive in situ hybridization study of Epstein-Barr virus-encoded RNA 1 (EBER-1) in lymph nodes, with lymphocytes infiltrating the liver and tissue from ethmoid sinus surgery 3 years before his presentation with obvious lymphoproliferative disease, suggested EBV association in lymphoproliferative disorder of the NK cell.

CASE REPORT

A 39-year-old Japanese male had recurrent upper respiratory infection, chronic sinusitis, and rhinitis, and he had received ethmoidectomy and tonsillectomy 3 years prior to admission. He was admitted to hospital because of fever in February 1987. Fever subsided with antibiotics, but he developed recurrence of fever and cervical lymphadenopathy and was referred to our department. Body

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Received for publication 8 June 1995; Accepted 8 May 1996.

temperature was 37.5°C. Examination showed several cervical lymph nodes that were 10 mm in diameter, and nontender hepatomegaly 1.5 cm below the right costal margin. The spleen was not palpable. Blood count revealed that hemoglobin was 11.8 g/dl, platelets were 356×10^9/l, and the white blood cell count was 6.17×10^9/l. Large granular lymphocytes were not present in the peripheral blood. The biochemistry profile was unremarkable. Computed tomography of the larynx revealed stuffing of each sinus with soft-density masses and mass invasion of the nasopharynx. He was treated with antibiotics, but his fever did not subside, and hence prednisolone, 40 mg/day, was started. Fever and cervical swelling subsided, but with reduction of prednisolone to 10 mg/day, he became febrile. In June, he developed jaundice, generalized lymphadenopathy, and hepatosplenomegaly, and he exhibited increase of aspartate aminotransferase and alanine aminotransferase. After the third biopsy of the lymph nodes, he was treated by chemotherapy with vincristine, cyclophosphamide, prednisolone, and adriamycin, but he died from hepatorenal insufficiency and ileus in July 1987. Diagnosis of biopsy specimens of lymph nodes performed three times was atypical lymphadenopathy with follicular depression, immunoblastic proliferation, and focal necrosis with eosinophilia in paracortex. Table I shows the surface marker studies of lymphocytes taken from biopsy specimens of lymph nodes and peripheral blood in June, 1987. Table II shows the serum samples assayed for antibodies to EBV capsid antigen (VCA) (IgG, IgM, and IgA), early antigens (EA) (IgG, IgM, and IgA), and EBNA (Epstein-Barr nuclear antigen) (IgG) by immunofluorescence. T-cell receptor gene-rearrangement and cytogenetic studies were not performed.

MATERIALS AND METHODS

Immunologic Studies

Mononuclear cells from heparinized peripheral blood and lymph node biopsy specimens were isolated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Rosette-forming capacities (RFC) with sheep erythrocyte (E-RFC), complement (erythrocyte-antibody-complement) (EAC-RFC), and IgG-Fc (erythrocyte antibody) (EA γ-RFC) were assayed according to standard techniques [7,8]. Immunophenotyping was performed on mononuclear cells by indirect immunofluorescence with a series of monoclonal antibodies (MoAbs) and fluorescein isothiocyanate (FITC)-conjugated goat $F(ab')_2$ fragments of anti-mouse IgG, using a Cytofluorograf System 50-H (Ortho Diagnostic Systems, Raritan, NJ). Surface phenotypes of lymph node biopsies and necropsy specimens from the liver were assessed on paraffin sections by the avidin-biotin immunoperoxidase technique. Monoclonal antibodies CD2 (OKT11), CD3 (OKT3), CD4 (OKT4), CD1a (OKT6), CD8 (OKT8), and CD11b (OKM1) were purchased from Ortho Pharmaceuticals (Raritan, NJ). CD5 (Leu1), CD7 (Leu9), CD16 (Leu11), CD56 (Leu19), CD57 (Leu7), and CD25 (anti-Tac) were obtained from Becton Dickinson (Mountain View, CA). CD19 (B4) and CD20 (B1) were obtained from Coulter Electronics (Hialeah, FL), and HLA-DR (LN-3) was from Nichirei (Tokyo, Japan). CD45RO (UCHL-1), CD20/cy (L26), LMP-1 (latent membrane protein of EBV), and ZEBRA (*Bam*HI Z fragment, Epstein-Barr-replication activator) monoclonal antibodies were from DAKO (Copenhagen, Denmark). LMP is known to be the protein produced in latent EBV infection, and ZEBRA originates from the BZLF1 (*Bam*HI Z left frame 1) sequence and is known to be involved in the switching of EBV from a latent to a lytic (productive) cycle [9].

In Situ Hybridization

Paraffin sections were mounted on aminopropyltriethoxysilane-coated slides (DAKO). Deparaffinized sections were incubated in 0.2 M hydrochloric acid for 10 min, washed in TBS (0.05 M Tris-buffered saline, pH 7.6), and digested with 10 μg/ml proteinase K. FITC-labeled oligonucleotide probe complementary to Epstein-Barr virus-encoded RNA (EBER)-1 (DAKO) was hybridized with histologic sections for 2 hr at 37°C, covered by cover glasses. The slides were then rinsed in TBS and incubated with alkaline phosphatase-labeled anti-FITC rabbit F (ab′) antibody. After rinsing with TBS, the slides were incubated with BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazorium) solution for 60 min. In situ hybridization was also performed with FITC-labeled antisense oligonucleotide complementary to the *Bam*HI H left frame 1 (BHLF1) oligonucleotide (DAKO). The EBER1 gene is known to be highly transcribed in latently infected cells (10^7 transcripts/cell), and to remain nonpolyadenylated in nuclei as ribonucleoproteins resistant to formalin fixation [10]. In contrast to EBER genes, the BHLF1 sequence is only transcribed in cells with EBV in the lytic cycle [10]. Counterstaining was performed with Mayer's hematoxylin solution or methyl green.

Double-labeling immunohistochemical and in situ hybridization studies were performed on formalin-fixed paraffin-embedded sections. Immunohistochemical studies using avidin-biotin peroxidase and mouse monoclonal antibodies were performed first, followed by in situ hybridization studies.

Polymerase Chain Reaction (PCR)

PCR was performed using oligonucleotide primers (primer 1, GGCGCACCTGGAGGTGGTCC; primer 2, TTTCCAGCAGAGTCGCTAGG) specific for the EBV lymphocyte-determined membrane antigen (LYDMA)

TABLE I. Surface-Marker Analysis and NK Activity of the Patient*

Marker	4/16, first biopsy, LND (left cervical)	5/9, second biopsy, LND (right cervical)	6/26, PBL	6/30, third biopsy, LND (right inguinal)
E-RFC	57	69	70	75.5
EAC-RFC	6	3	2	4.5
EA γ-RFC	9	10.5	10	7
CD3	40	15.5	70.2	45
CD4	26	20	22.9	37
CD1a	n.t.	2	n.t.	n.t.
CD8	17	5	45.3	13.8
DR	49	90.5	n.t.	54
CD11	12	5	n.t.	n.t.
CD20	12	6	n.t.	5.8
CD19	15	5	n.t.	n.t.
CD2	73	85	91.3	61
CD5	40	35	n.t.	49
CD7	55	19	n.t.	40
CD57	n.t.	8	37.1	15
CD56	n.t.	84.5	44.5	65
CD16	n.t.	1.5	35	22
CD25	n.t.	38	n.t.	n.t.
NK activity (%)	n.t.	−2.5	1.7	n.t.

*Numbers represent percentage of lymphocytes positive for the marker. LND, lymph node; PBL, peripheral blood lymphocyte; E-RFC, rosette-forming capacity with sheep erythrocyte; EAC-RFC, rosette-forming capacity with complement (erythrocyte-antibody-complement); EA γ-RFC, rosette-forming capacity with IgG-Fc (erythrocyte antibody); NK activity, natural killer cell activity; n.t., not tested.

TABLE II. EBV Serology of the Patient*

Date	VCAIgG	VCAIGM	EAIgG	EAIgM	EBNA
April 16, 1987	160	<10	320	10	40
May 15, 1987	640	<10	320	10	40
June 9, 1987	1,280	<10	160	10	160
June 30, 1987	160	<10	40	<10	<10

*VCA, viral capsid antigen; EA, early antigen; EBNA, Epstein-Barr nuclear antigen.

gene, which is composed of variable numbers of tandem repeats [11]. The size of this heterogeneous region is characteristic for a given EBV isolate, and can vary between EBV isolates [12]. One μl of DNA was amplified, as recommended by the manufacturer, in a DNA thermal cycler (Cetus, Norwalk, CT) for 30 cycles: 1 min denaturation at 94°C, 2 min annealing at 60°C, and 3 min extension at 72°C. The reaction products were examined by direct visualization in ethidium bromide-stained ultraviolet-illuminated agarose gels. DNA for PCR was extracted from formalin-fixed paraffin-embedded material, using a method described by Mies [13]. Paraffin blocks were cut into 5-μm-thick sections and collected in Eppendorf tubes. The sections were deparaffinized by extraction with xylene, and then washed with ethanol. Proteinase K digestion was performed for 3 days at 37°C with Tris-NaCl-EDTA (TNE) buffer. Boiling inactivates the proteinase, and centrifugation provides a supernatant that contains DNA suitable for amplification. The integrity of the DNA extracted from the tissue sections was confirmed by amplifying a 140-bp fragment of exon 6 of the E-cadherin gene, using standard PCR.

Natural Killer Cell Activity

Table I shows NK activity measured by a cytotoxicity assay using ^{51}Cr-labeled K562 cells as the target [14]. The effector and target cell ratio was 40:1, and incubation time was 4 hr.

RESULTS

Characterization of NK Cells

May-Giemsa staining of large granular lymphocytes, which appeared in the peripheral blood in the latter part of the patient's clinical course, showed several azurophilic granules in the cytoplasm (Fig. 1). Biopsy specimens from the lymph node showed loss of lymph follicles and infiltration of medium-sized atypical cells (Fig. 2). Transmission electron microscopy of the cells isolated from the lymph node biopsy specimens showed an irregularly shaped nucleus, with nucleoli and wide cytoplasm containing Golgi apparatus and mitochondria (Fig. 3). Several electron-dense granules were present. Parallel tubular arrays were not present.

The phenotype of mononuclear cells from lymph node biopsy specimens and peripheral blood from the leukemic

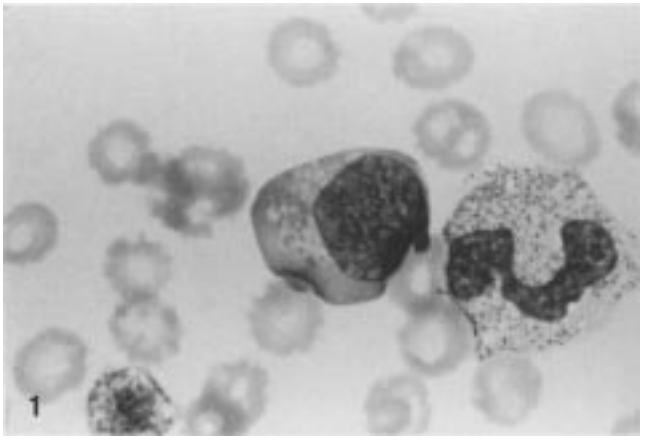


Fig. 1. May-Giemsa staining of a peripheral blood smear on July 1, 1987 showed large granular lymphocyte with azurophilic granules (×1,000).

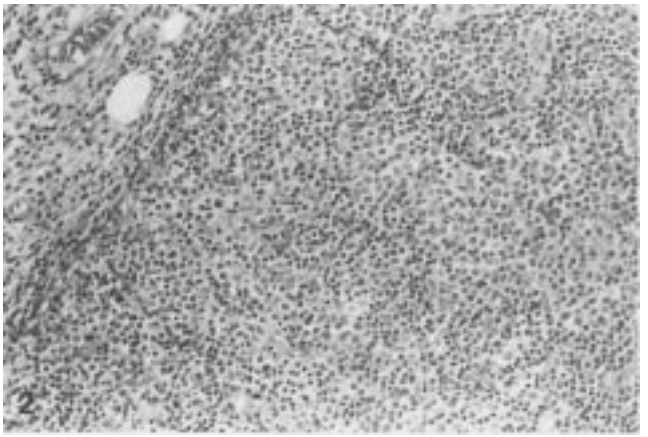


Fig. 2. Biopsy specimen from a lymph node showed loss of lymph follicles and infiltration of medium-sized atypical cells (×100).

phase was CD2+, CD3−, CD4−, CD8−, CD11b−, CD16+, HLA-DR+, CD56+, and CD57− (Table I). The results of NK activity are also shown in Table I, which was markedly decreased compared to normal controls (59.4 ± 13.0%).

EBV Serology

EBV serology results are summarized in Table II and indicate reactivated EBV infection with elevated VCAIgG and EAIgG titers.

In Situ Hybridization

Direct evidence for the presence of EBV within NK cells was sought by in situ hybridization. Lymph node biopsy specimens showed strong labeling with an oligonucleotide probe to EBER-1 (Fig. 4a), and the control study performed from the same lymph node biopsy specimen without an oligonucleotide probe did not show any

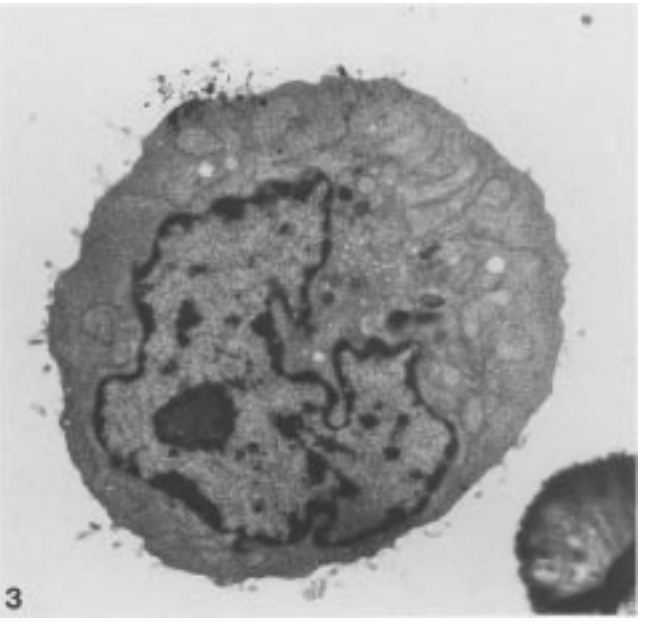


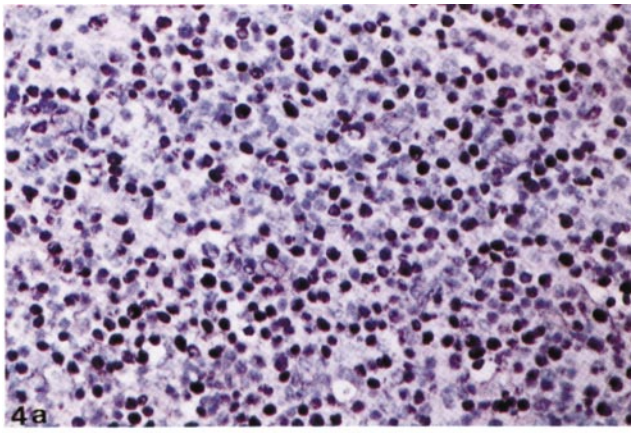
Fig. 3. Transmission electron microscopy of cell isolated from lymph node biopsy specimen showed an irregularly-shaped nucleus with nucleoli and wide cytoplasm containing Golgi apparatus, mitochondria, and several electron-dense granules ×5,000).

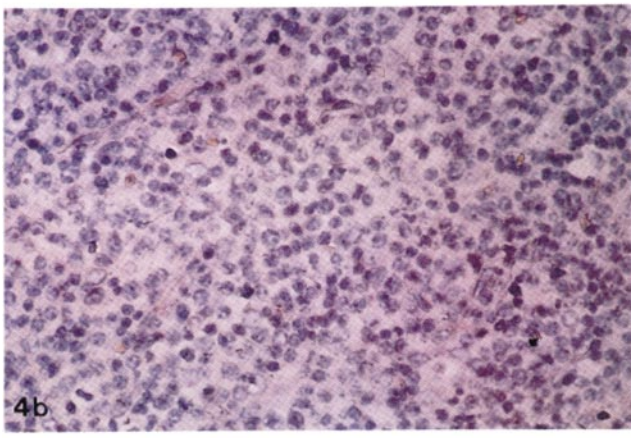
labeling (Fig. 4b). The pathology specimen obtained at the time of the patient's ethmoid sinus surgery, 3 years before he presented with obvious lymphoproliferative disease, was reviewed, and sinus involvement by abnormal lymphocytes was confirmed. In situ hybridization study with EBER-1 revealed obvious labeling (Fig. 4c). A necropsy specimen from the liver also revealed definite labeling within infiltrating mononuclear cells (Fig. 5). Double-labeling immunohistochemical and in situ hybridization studies revealed that EBER-1-positive cells were positive for HLA-DR (Fig. 6), and negative for CD20/cy and CD45RO (data not shown) in the lymph node. EBER-1-positive mononuclear cells infiltrating the liver were also HLA-DR-positive (Fig. 7). In situ hybridization with antisense oligonucleotide specific to BHLF1 revealed positive signals in a small number of cells in the lymph node specimens (Fig. 8).

EBV Protein Expression

Immunohistochemical staining with monoclonal antibody CS1-4 (LMP-1) and ZEBRA protein were negative in lymph node biopsy specimens.

In the PCR study using primers specific for the LYDMA gene, a single DNA band was detected from the DNA extracted from a lymph node biopsy specimen, indicating a monoclonal disorder of this natural killer cell lymphoproliferative disorder (Fig. 9).





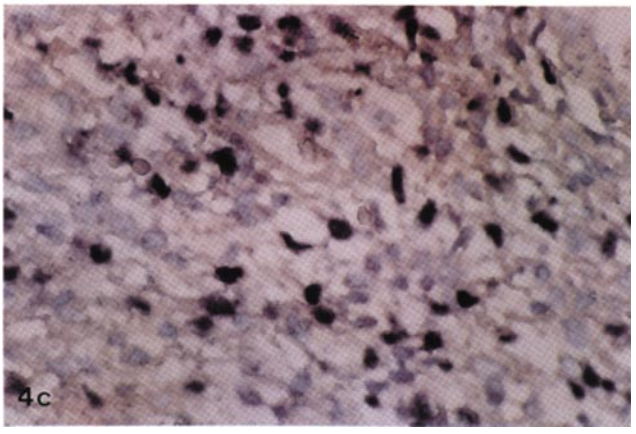


Fig. 4. a: In situ hybridization of a lymph node with an oligonucleotide probe to EBER-1. Mononuclear cells showed strong labeling with oligonucleotides (×200). b: Control study of in situ hybridization performed from the same lymph node biopsy specimen without an oligonucleotide probe did not show any labeling (×200). c: Pathology specimen from ethmoid sinus revealed definite labeling with an oligonucleotide probe to EBER-1 (×200).

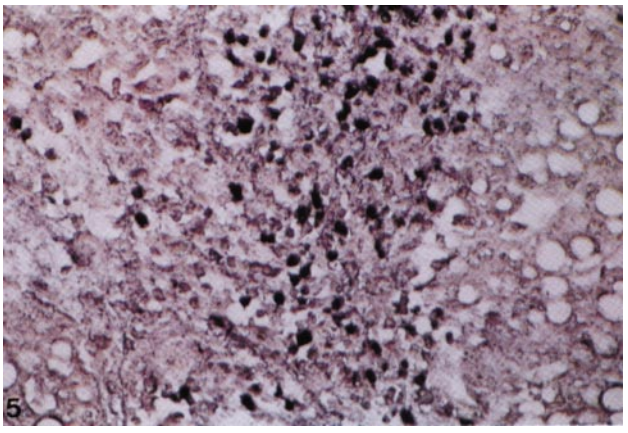


Fig. 5. Necropsy specimen from the liver revealed strong labeling of infiltrating mononuclear cells. However, hepatic cells did not show labeling with oligonucleotides (×200).

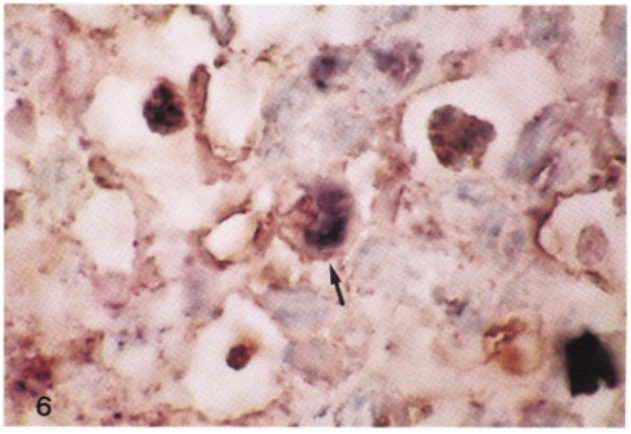


Fig. 6. Double-labeling with EBER-1 and monoclonal antibody of lymph node biopsy specimen. The cytoplasm of the cell in the middle (arrow) stained brown with HLA-DR, and the nucleus of the cell stained blue, indicating EBER-1 hybridization with oligonucleotides. Immunohistochemistry was performed first, followed by in situ hybridization (×1,000).

DISCUSSION

In light of the evidence that clonal large granular lymphocyte (LGL) proliferation may be either CD3+ (T-cell origin) or CD3− (NK cell origin), it is proposed that the following terminology be adopted: 1) T-LGL leukemia characterized by clonal CD3+ LGL proliferation, and 2) NK-LGL leukemia, characterized by clonal CD3− LGL proliferation [4].

An aggressive clinical course was observed in our patient, terminating in death from hepatorenal insufficiency after 5 months from his last admission, despite multiagent chemotherapy. The surface markers of pathologic cells from our patient were CD2+, CD3−, CD16+, CD56+, and HLA-DR+. We believe that his pathologic cells were CD16+, because 35% of peripheral blood lymphocytes (PBL) expressed CD16 on 6/26, as did 22% of cells from 6/30 in the lymph node biopsy. These percentages are

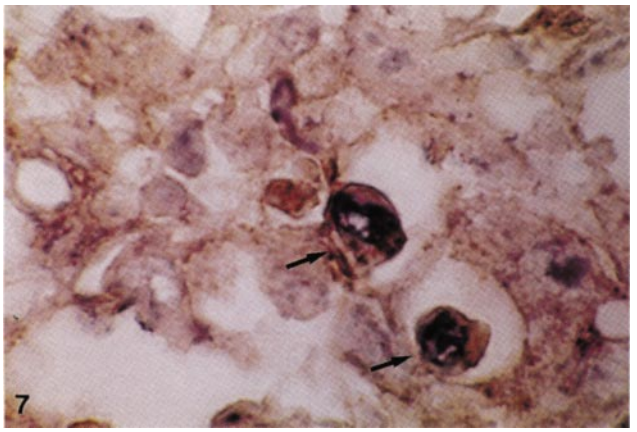


Fig. 7. In situ hybridization and immunohistochemistry of necropsy specimen from the liver. The cytoplasm of infiltrating cells (arrows) stained brown with HLA-DR, and nuclei of these cells stained blue, indicating presence of EBER-1 (×1,000).

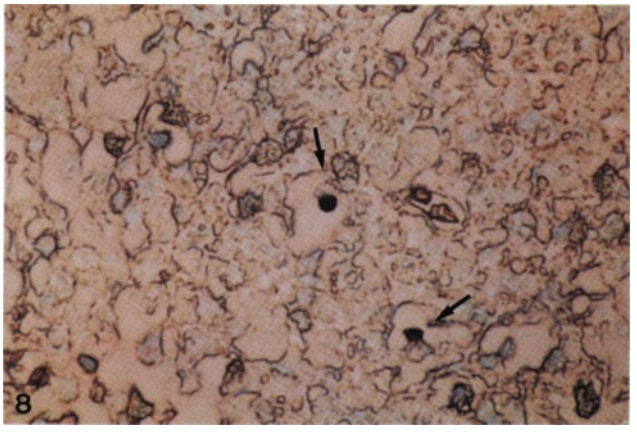


Fig. 8. In situ hybridization with antisense oligonucleotide to BHLF1 revealed positive signals in a small number of cells (arrows) in the lymph node (×400).

considerably higher than would be expected from normal NK cells in these sites, and suggest that CD16 was in fact expressed by neoplastic cells. CD3 positivity in 40%, 15.5%, 70.2% (peripheral blood), and 45% of lymphocytes obtained either by lymph node biopsy or by peripheral blood analysis must represent the presence of CD4+ or CD8+ lymphocytes. Accordingly, the patient had lymphoproliferative disorder of NK cells, although a pathologic diagnosis of malignant lymphoma was not made. On admission, he did not have pathologic lymphocytes in peripheral blood or in bone marrow. However, pathologic cells appeared in his peripheral blood about 20 days before death. NK cell-type LGL patients tend to have an aggressive clinical course [15–22], while patients with CD3+ LGL have a stable course without any treatment [14,23].

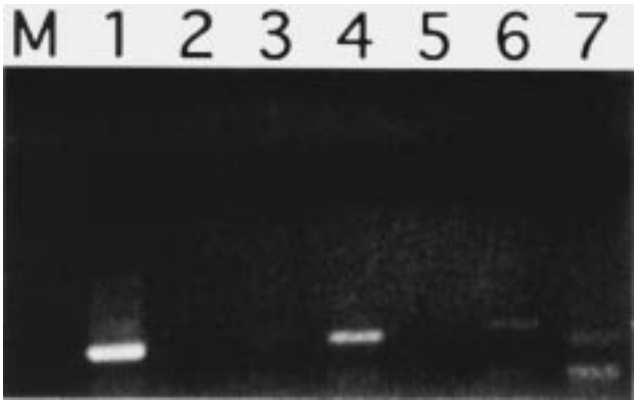


Fig. 9. PCR products amplified from a region of the EBV LYDMA gene. M, marker; lane 1, Raji cell, EBV+; lane 2, water; lane 3, sample from this patient's second lymph node biopsy; lane 4, sample from this patient's third lymph node biopsy; lane 5, sample from this patient's necropsy specimen from the liver; lanes 6 and 7, samples from Hodgkin's disease patients. Samples from Raji cell line (lane 1) and patient's lymph node (lane 4) showed a single band, while samples from Hodgkin's disease patients revealed two bands.

Kawa-Ha et al. [5] have implicated EBV infection in the pathogenesis of NK-LGL leukemia from Japanese patients, because they found clonal integration of EBV in leukemic cells. Chan et al. [24] could not find EBV DNA in any of 5 patients studied. Positive in situ hybridization with EBER-1 in lymph node biopsy specimens and necropsy specimens from the liver confirmed EBV involvement in our patient. The positive staining of cytoplasm of EBER-1-positive cells in the lymph node and liver with monoclonal HLA-DR antibody suggested the infection of NK cells with EBV. As CD2 and CD56 monoclonal antibodies are known to be unsuitable for formalin-fixed paraffin-embedded specimens, monoclonal HLA-DR antibody was the only one available for double-labeling with EBER-1 in this case. When the VCAIgG titer is elevated to .320-fold dilution or the EAIgG titer is elevated to .10-fold dilution, these results are considered to indicate reactivated EBV infection [25]. The results of a serologic study of EBV in this patient suggested reactivation of latent infection with elevation of the VCA IgG titer to .1,280-fold dilution and the EA IgG titer to .320-fold dilution. Marked decrease in the level of EBNA antibody titer to ,10-fold dilution correlated with disease progression [26]. Positive in situ hybridization studies with antisense oligonucleotide to BHLF1 mRNA suggested reactivation of EBV, but lytic infection-specific protein ZEBRA was negative. Under conditions in which EBV gene products are activated, the two major latent gene products, Epstein-Barr virus-encoded RNAs and Epstein-Barr virus nuclear antigen mRNA, are expressed at a constitutional level [27]. Markedly decreased NK activity of lymphocytes from the

lymph node and peripheral blood suggests that the NK cells infected with EB virus lost their NK activity.

EBER-1 and EBER-2 genes are known to be highly transcribed in latently infected cells (10^7 transcripts/cell) [10]. The stability and considerable abundance of EBER-1messenger RNA must have allowed detection by in situ hybridization. Amplification of the LYDMA gene sequence of the patient's lymph node biopsy specimen revealed a single band, indicating that EBV detected in the lymph node originated from single EBV [11,12], a monoclonal proliferation of a natural killer cell.

These results of in situ hybridization studies, PCR study, immunohistochemical studies, and serologic study suggest a close relationship between EBV infection and development of NK cell lymphoproliferative disorder. Because abnormal lymphocytes were present and EBER-1 was positive in the pathology specimen from ethmoid sinus surgery 3 years before the patient presented with obvious lymphoproliferative disease, it would suggest that the patient had a relatively prolonged "indolent" phase of disease followed by a transformation to the aggressive phase. In aggressive cases of EB virus-associated NK lymphoproliferative disorder, a different approach from conventional chemotherapy may be required.

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